FABRICATION OF COLLAGEN SCAFFOLDS FOR RECONSTRUCTION OF URINARY TISSUES

By

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ABSTRACT

Tissue engineering approaches utilize scaffolds with or without cells to reconstruct, repair, or regenerate lost tissues. These scaffolds are created using either natural or synthetic biomaterials or their composites. Although each of these biomaterials has its own advantages and disadvantages, collagen-based scaffolds are a popular choice in tissue engineering because collagen is a major protein component of extracellular matrices (ECM). Despite providing the favorable biological environment for cells to recreate tissues, collagen-based scaffolds suffer limited mechanical properties, mostly due to limitations in laboratory-based fabrication methodologies.

To overcome this and increase a scaffold's mechanical property, compression molding of collagen, which leads to a dense collagen material, was developed; however, it is confined to creating thick sheets and films. We therefore aimed to develop a biofabrication method that can mold collagen scaffolds into tubular and hollow structures. It is particularly important for genitourinary tissue engineering, where tubular and hollow scaffolds are needed for tissue reconstruction.

We utilized Solidworks^{TM} software to design hollow and tubular molds and 3D print them using a biodegradable material. We further devised a biofabrication chamber to produce tubular and hollow collagen scaffolds. We also fabricated collagen discs that are potentially usable as a patch for partial graft applications and tested their mechanical properties by measuring its breaking point from suture and tensile tests, as well as observing its performance during a stress-strain hysteresis loop.

We plan to devise methods to improve the mechanical strength of collagen, starting with various physical and chemical modifications. The next steps involve a current project with the goal of developing a urodynamic chamber and a simulation-based software program to evaluate the performance of scaffolds under urinary flow conditions. The future steps will be evaluating these scaffolds in small animal models to study their *in vivo* biomechanical performance.

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CHAPTER 1: INTRODUCTION

1.1 Bladder Cancer and Current Treatments

Bladder cancer is the fourth most common cancer in men. It affected about 68,000 adults in the United States in 2017, and there are projected to be 81,900 new cases and 17,240 deaths from bladder cancer in 2018. [1,2] It most frequently affects older males but can also affect women and younger people. It usually starts when the urothelial cells that line the inside of the bladder start to grow excessively. However, it can occur in other areas of the urinary tract, such as ureters or the urethra. [3] It hinders quality of life and requires extensive diagnosis, treatment, and medical care. [4]

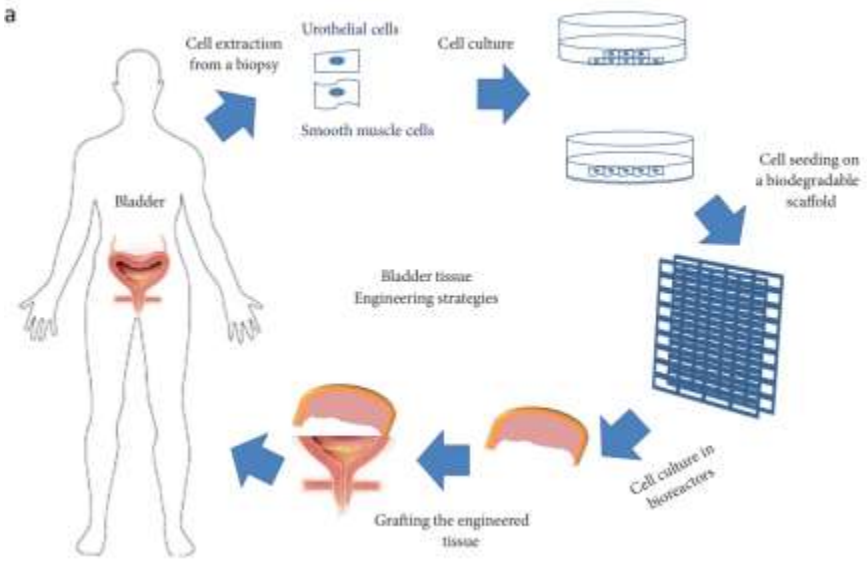
Bladder cancer and other urinary tract defects may require total replacements depending on the severity. Reconstructive procedures frequently use gastrointestinal segments, which is not ideal for bladder procedures because gastrointestinal tissue absorbs the solutes that bladder tissue wants to excrete. This pervasive problem led scientists to research alternative methods for bladder repair or replacement. [5]

1.2 Introduction to Urinary Tissue Engineering

Tissue engineering uses highly porous artificial extracellular matrices, otherwise known as scaffolds, to fill tissue voids, provide structural support, and deliver cells and growth factors that can form tissues in three dimensions in the body upon transplantation. Biologically active factors and DNA are essential in contributing to these objectives. Among the existing possibilities, three-dimensional (3D) scaffolds have been one of the most effective and analyzed options. The two

main uses of scaffolds in tissue engineering are as a cell support device for *in vitro* seeding and as a growth factor/drug delivery device. Many of these scaffolding procedures are often combined to increase effectiveness. [6] A couple of the biggest ongoing challenges in tissue engineering are the improvements to mechanical strength of scaffolds and interconnection channels that allow cell growth on scaffolds. [7]

Urinary tissue engineering focuses on treatment methods for the bladder, urethra, and ureters. In urology, one of the first goals was to create a bladder replacement. The first prototypes used synthetic non-biodegradable materials like silicone, rubber, polypropylene, and polytetrafluoroethylene. However, they were prone to infection and caused foreign body reactions. Present day models emphasize the design of implantable scaffolds to mimic the physiology and function of healthy urinary components. Although one of the primary goals remains to create a viable bladder replacement, methods are constantly optimized by new findings. Procedures for the bladder and tubular scaffolds (e.g. ureters, urethra) follow a general formulation **(Figure 1)**. [8] Scientists continue to advance and improve medicine with the growing number of tissue engineering protocols, with the objective of achieving widespread clinical applicability. [9]



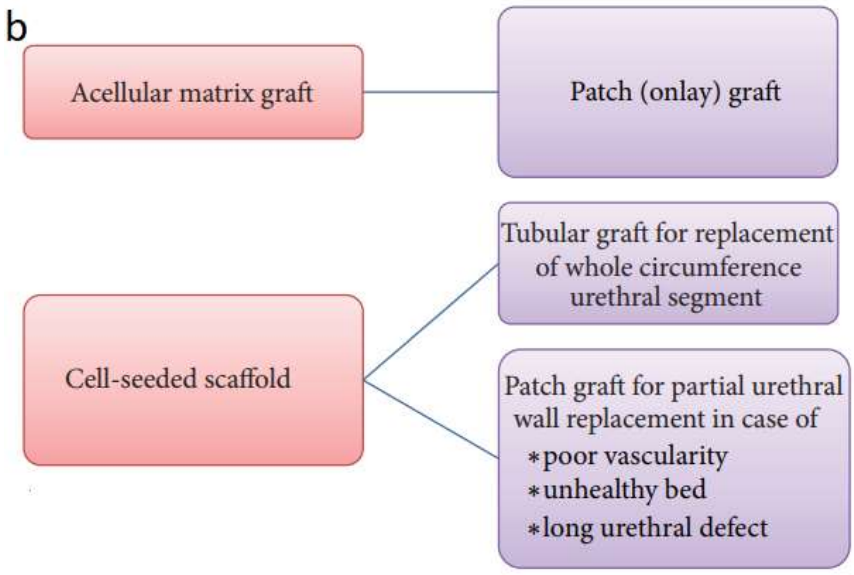


Figure 1: General strategy formulation for urinary tissue engineering. The bladder has one general route of work that uses cell-seeded scaffolds (a), while tubular scaffolds have several options and can be acellular depending on the application (b). [8]

3D printing has been prevalent in medical fields that need unique replacement parts for repair, such as ophthalmology and orthopedics. The field is still undergoing rapid progress including urology. Furthermore, 3D printing in urology is more complex with focuses on solid and hollow viscous organs, such as the bladder, kidney, and ureters. Within urology, there is potential for it to grow as a surgical planning and education tool, medical device production method, and for the bioengineering of bioactive materials **(Figure 2)**. Significant advancement would allow urologists to create viable replacement 3D-printed scaffolds to restore function to a patient's urinary tract and improve their quality of life. [10]

Figure 2: Applications of 3D printing in urology. A 3D model of a prostate tumor from an MRI is used for educating and training students for procedures (a). 3D printing can also be used for bioengineering of urological components, such as a kidney scaffold (b). Reprinted with permission from [10]. © 2018, Springer Nature.

1.3 Biomaterials for Urinary Tissue Engineering

1.3.1 Selection Criteria

Biomaterials are an integral aspect of tissue engineering. They contribute to a number of factors and play an important part in the viability of a scaffold. A biomaterial must be both biocompatible and mechanically stable to fit the minimal requirements. The ideal biomaterial for bladder and urethral reconstruction should allow for even and constant attachment of mature epithelial cell layer on the luminal surface and keep multiple cell layers of smooth muscle cells on the outside. [8]

Biodegradability is important because the byproduct production of toxic chemicals would be harmful to the host. The degradation rate and byproduct concentration within tissues must be below the threshold of negative effect to allow for optimal tissue regeneration. Controlling these factors mitigates the risk of a foreign body response or inflammation that can occur when implanting a scaffold in the body. The degradation rate is also important to the longevity and functionality of a scaffold because it can lead to the need for surgical replacement, which can be financially and physically taxing. [5]

The biomaterial of choice should also be able to regulate cell behavior to promote new tissue development. This is regulated by microenvironment interactions, mainly with cell-adhesion ligands. A scaffold should be able to support cellular activity for multiple cell types. Whether they are inherent or incorporated in the biomaterial, cell-adhesion-promoting factors are important to possess to control ligand-induced cell receptor signaling processes. An effective biomaterial should allow for easy tissue development to restore function to the body and system. [5]

It must also provide sufficient mechanical strength and prevent premature mechanical collapse before new tissue formation *in vivo*. For the urinary tract, the main forces are urine filling and emptying, flow, and storage. Mechanical support for biomaterials must be maintained until the tissue has developed enough strength to support itself. [5] If a scaffold is stitched into place, it is important to ensure no tearing will occur at any points of stitching. [8]

There are three general classes of biomaterials for urinary tissue engineering: natural biomaterials (e.g. collagen, alginate), synthetic biomaterials (e.g. polyglycolic acid, silicone), and acellular tissue matrices (e.g. bladder submucosa, small-intestinal submucosa). [5] Natural and synthetic biomaterials are paramount to this project and will therefore be covered. They have been frequently explored in other projects and this project covers the potential benefits of both types.

Polymer blends are also important to consider in this case because the benefits of combining natural and synthetic biomaterials into a copolymer blend can be very useful for the purposes of urinary tissue engineering.

1.3.2 Natural Biomaterials

Natural biomaterials are naturally occurring materials and contain many inherent proteins and factors, which makes them more biocompatible and allows them to support cell adhesion, migration, proliferation, and differentiation. They are biodegradable and capable of supporting cell growth and tissue remodeling, making them ideal for replacement or restoration of the function and structure of damaged organs and tissues. Natural materials do not trigger a serious host response, which simplifies the implantation of a scaffold. They are frequently used for nerve, skin, cartilage, and bone repair. Of the many options available, alginate, chitin, chitosan, collagen, glycosaminoglycans, and starch are the most used natural biomaterials. [5, 7, 11]

Natural biomaterials are often scrutinized because of several deficiencies. They are subject to large batch-to-batch variation, which can limit use for wider applications. Potential impurities can cause immunogenicity and risk damaging the host upon implantation of a scaffold. They are also mechanically weak, which reduces their viability for *in vivo* performance. Although there are other shortcomings, these are the main points of concern for choosing natural biomaterials for a tissue engineering scaffold. [7]

1.3.3 Synthetic Biomaterials

Synthetic biomaterials are cost-efficient to mass produce and easy to shape to a desired 3D shape. Many material properties are easily tunable, which makes them tailorable to specific functions. The degradation rate, mechanical properties, microstructure, and porosity are all important properties of urinary tissue engineering scaffolds and can be easily controlled. For any tissue engineering protocol that requires 3D printing, synthetic biomaterials are an excellent choice because of the simplicity of the process. [8, 12] Polyglycolic acid (PGA), polylactic acid (PLA), poly(lactic-co-glycolic acid), and poly(ε-caprolactone) are some of the most frequently used synthetic biomaterials and are also often used to make 3D scaffolds. [5, 7]

Synthetic biomaterials are mainly disadvantageous because they are foreign compounds, which will trigger a host response. The difference in composition and structure makes them less biocompatible and biodegradable. Tissue remodeling is less inducible, which reduces *in vivo* viability. While the mechanical strength can be useful to have for load-bearing applications, it can be a hindrance for mechanical compliance with soft tissue or blood vessels. [7] These issues often result in the need for synthetic materials to be paired with a natural material for biomedical applications. [11]

1.3.4 Polymer Blends

A polymer blend is a mix of natural and synthetic biomaterials with unique mechanical and structural properties. Synthetic biomaterials are easier to use in biomedical applications, but natural biomaterials are more essential because of their biocompatibility. To address this problem, a blend takes multiple biomaterials to make a compound with improved biocompatibility,

degradation, and mechanical properties compared to their individual components. A blend can use multiple natural or synthetic types, but the most common blends feature a mix of a natural and synthetic biomaterial. [13]

Blends are used for desired properties and optimizing them for a desired function. The tunability of synthetic materials helps to improve the deficiencies of natural materials, while the biocompatibility of natural materials makes the application of synthetic materials less worrisome. For urinary tissue engineering specifically, there is no biomaterial that can fulfill the criteria mentioned before. Natural and synthetic scaffolds have their respective drawbacks, but a blend could address and attempt to mitigate those problems. [5,7,12,13]

1.4 Collagen

1.4.1 Structure

Collagen is a major protein in the extracellular matrix, and the most abundant protein of connective tissue in animals. [11] There are at least 16 types of collagen, with type I being one of the most abundant types of collagen in animals and the first to be characterized. Its main purpose is to help tissues withstand stretching, which is a shared purpose across all types of collagen. It has a right-handed triple helix structure that includes a prolific amount of the amino acids glycine, proline, and hydroxyproline **(Figure 3a)**. These amino acids set up the frequently occurring Gly-Pro-X sequences, where X represents any amino acid. Each of these amino acids has a specific function in the collagen structure. Glycine is special because its hydrogen side chain is the only side chain that can fit into the center of the triple stranded helix. The hydrogen bonds that link the peptide bond amine of glycine with a peptide carbonyl of an adjacent polypeptide help to hold the

three chains together **(Figure 3b, 3c)**. Lastly, the angle of the C-N bond between a peptide and proline or hydroxyproline allows polypeptide chains to fold into a helix so that three chains can twist to form the triple-stranded helix. The peptidyl-proline linkages stabilize the helical structure of collagen. [14, 15]

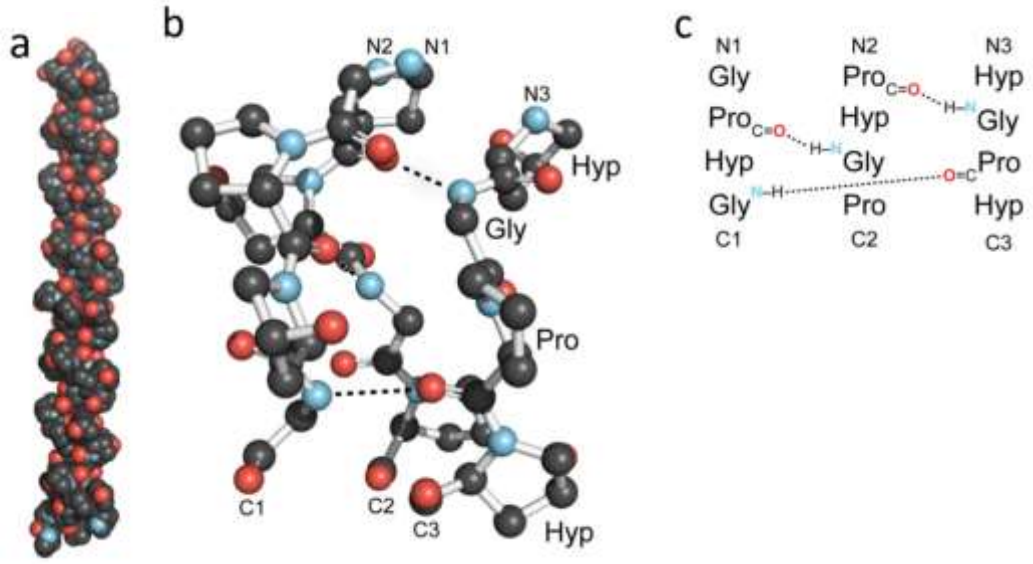


Figure 3: Structure of the collagen triple helix. The helical structure is apparent in the coiling carbon (black) chain (a). Nitrogen (red) and oxygen (blue) atoms are exposed on the outsides of the chain to promote hydrogen bonding with other collagen chains. The triple helix is shown in structural form (b) and separated to show the bonds that hold the strands together (c). Reprinted with permission from [15]. © 2009, Annual Reviews.

Collagen chains are first produced as longer precursors with loose ends, known as procollagens. Loose ends are cleaved by procollagen proteinases to form individual triple helical proteins, otherwise known as tropocollagen. Tropocollagen monomers arrange to form fibrils that possess high tensile strength and can be modified easily to support more stress. Fibrillogenesis *in situ* occurs through two stages of self-assembly of microfibrils: nucleation and fiber growth. These stages are highlighted by the elongation and stabilization of collagen fibers based on the structure of tropocollagen monomers. Lateral interactions pack fibrils side-by-side in parallel bundles and promote the formation of fibers and networks found in bone and tissue *in vivo* **(Figure 4)**. [14, 16]

This is an important process in the formation of biological scaffolds, as it helps to stabilize triple helices and support stress in three dimensions. [15]

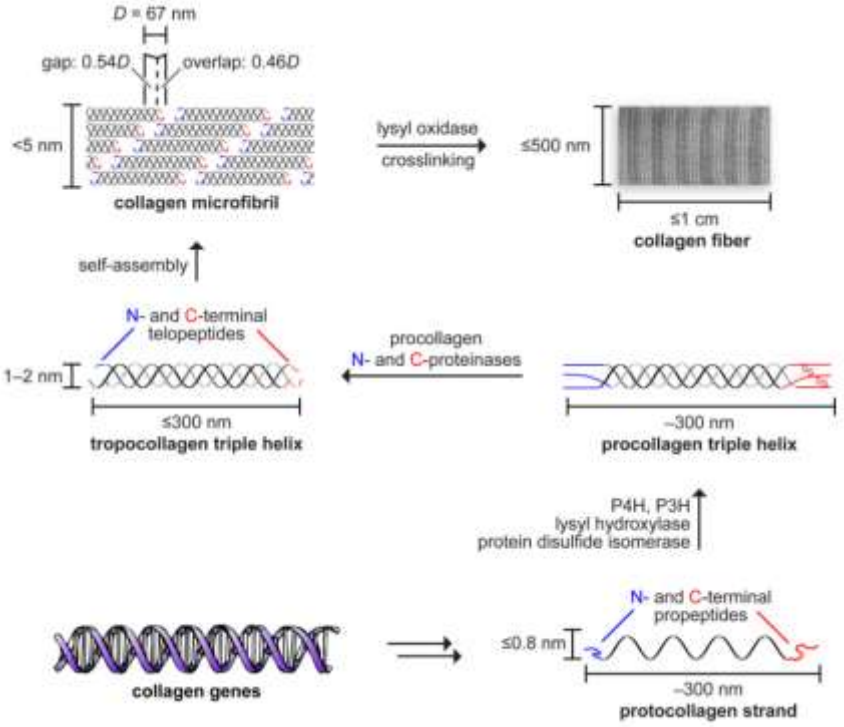


Figure 4: Synthesis of higher order collagen structures. The procollagen triple helix is cleaved by proteinases on the N- and C-terminal ends to form a tropocollagen monomer. These self-assemble by lateral interactions that pack monomers in a parallel order to form microfibrils, which can be cross-linked to form collagen fibers. Reprinted with permission from [15]. © 2009, Annual Reviews.

1.4.2 Biomaterial Application

Collagen is also a ubiquitous biomaterial that can form compact cross-linked solids or lattice gels. It exhibits time-dependent strain, making it a viscoelastic biomaterial. Its viscoelasticity is a result of its fibrillar structure. [17, 18] It exhibits minimal inflammatory and antigenic responses, and has been approved by the US Food and Drug Administration (FDA) for numerous applications. Intermolecular cross-linking reduced the degradation rate by limiting enzyme activity. Cross-linking can be accomplished through numerous physical (e.g. ultraviolet

radiation) and chemical (e.g. hyaluronic acid) modification techniques. The easiness of modification makes collagen an ideal choice for urinary tissue engineering. [5]

Collagen as a biomaterial has the same limitations as most natural biomaterials. It brings the risks of heterogeneity, immunogenicity, and loss of structural integrity during isolation. Collagen as a protein possesses good mechanical strength, but as a biomaterial for scaffolds it has poor mechanical performance. [7] An effective synthetic source of collagen and collagen-like proteins and fibrils has been observed as a potential solution to these problems. However, synthetic collagen would be difficult to use because of post-translational modification and the need for complex expression systems. [15]

Collagen is a seamless choice for urinary tissue engineering because of its biocompatability. It does not trigger a foreign body response since it is an abundant protein in the body. RGD (arginine-glycine-aspartic acid) cell adhesion sequences promote cellular activity and help to retain cellular phenotypes and activity for important cells, such as fibroblasts and chondrocytes. These sequences are important for urinary tissue engineering because they can help to promote cellular activity in a scaffold made from collagen. [5, 12]

CHAPTER 2: BIOFABRICATION OF COLLAGEN-BASED SCAFFOLDS FOR URINARY TISSUE ENGINEERING

2.1 Introduction to Collagen-Based Scaffolds for Urinary Tissue Engineering

Collagen-based scaffolds have been observed in numerous publications and clinical settings. They are already a very intriguing option in regenerative medicine, with numerous methods for production and characterization in existence. [19] Collagen is a great biomaterial for urinary tissue engineering scaffolds because of its biocompatibility, resistance to shear flow, and viscoelasticity that allows for constant stretching and relaxation. [18,20] Not all collagen-based scaffolds have performed well, and not all well-performing scaffolds have made their way to clinical implementation. However, the material properties and flexibility for modification make collagen a popular choice for the fabrication of a clinically successful scaffold for urinary tissue engineering. [21]

Collagen-glycosaminoglycan scaffolds (CGSs) promote partial organ regeneration due to their biological activity that blocks the healing response. This occurs from the binding of contractile fibroblasts through integrin-ligand binding, which blocks the generation of macroscopic contractile forces normally deployed to contract wounds in injured organs. Pore size is critical to the surface cell receptor ligand density. CGSs have been used for regeneration of skin, adult organs, and limb paralysis due to trauma. [19] In this case, collagen after chemical modification with a natural biomaterial created an effective biomaterial for partial organ regeneration.

Another reported use of collagen-based scaffolds from Atala et al. tested cell-seeded collagen and polyglycolic acid-collagen (PGA-collagen) scaffolds for bladder replacement

(Figure 5). Patients treated with PGA-collagen scaffolds and omental coverage showed increased compliance, decreased end-filling pressures, increased capacities, and longer dry periods over time. These positive results affirmed the improvement of the engineered bladder over time to better mirror the environment. [5] This example shows the effectiveness of collagen after chemical modification with a synthetic biomaterial.

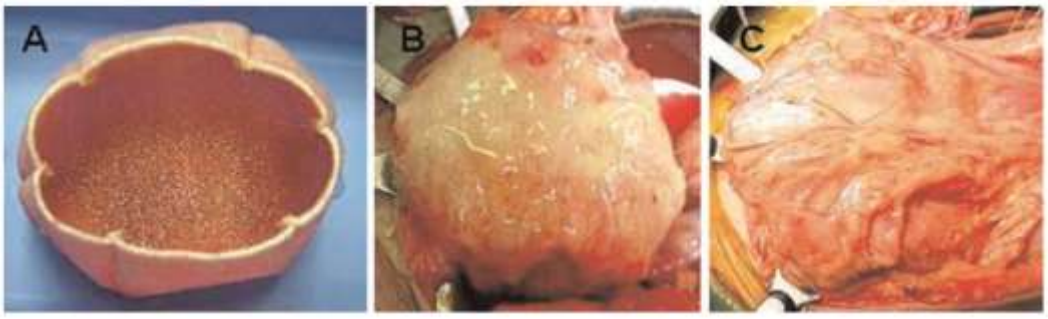


Figure 5: Construction and clinical use of a PGA-collagen scaffold. The cell-seeded scaffold is shown before implementation (A), surgically connected to the native bladder (B), and covered with fibrin glue and omentum (C). Reprinted with permission from [5]. © 2011, Oxford University Press.

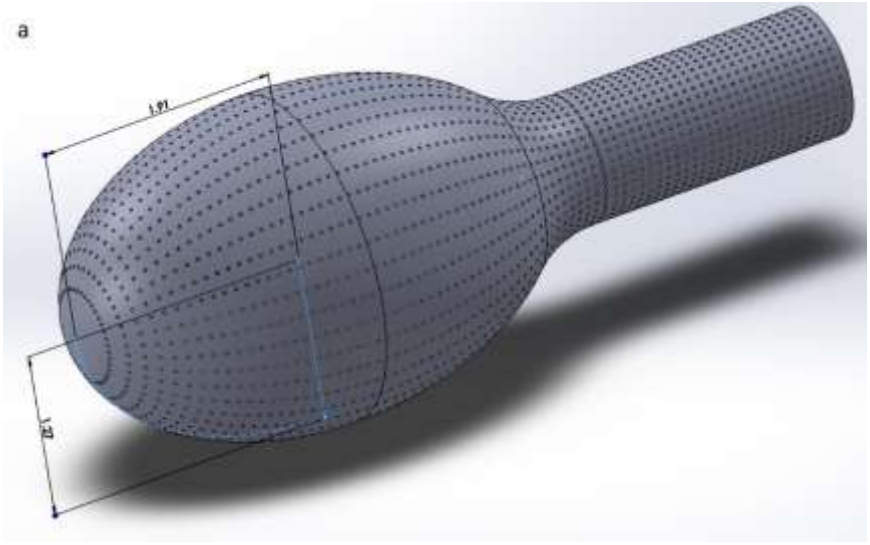
2.2 Design Consideration for Urinary Tissue Engineering Scaffolds

There are several key factors to consider when designing urinary tissue engineering scaffolds. One critical requirement is that the scaffold must be able to endure fluid pressure and allow lumen expansion with minimal change in fluid pressure. Of the factors that can affect burst pressure, material and wall thickness are a couple of the most important ones. Scaffolds also require high porosity and a high surface area to volume ratio. [5] These factors are easily manipulatable when using a 3D-printed mold because of the liberty in biomaterial choice and scaffold design. [20]

Material choice is critical because it requires consideration in multiple areas and affects downstream factors. Strength is crucial to the functional aspects and preventing the scaffold from

rupturing after implantation. Biocompatibility is just as important because of the cell seeding viability and foreign body response. Natural biomaterials are reasonable choices because of their biocompatibility and biodegradability, but mechanical strength is often lacking. Synthetic biomaterials share the opposite problem, as they possess the necessary strength but raise questions about biocompatibilty. As covered before, a blend may be the best answer to engineer a material that can endure high pressure and reduce the host response. [7, 13]

These primary considerations were applied to a computer-aided design (CAD) model of a bladder mold using SolidWorks™ **(Figure 6)**. Micropores were sized at 150 μm and separated diameter-to-diameter by 250 μm. The wall thickness was set at 0.15 cm. The radii of the balloon section were set at 1.27 cm (0.5 in) and 1.91 cm (0.75 in). The tube length was set at 2.54 cm (1 in) and the outer radius was set at 0.5 cm. This design is highlighted by the micropores that are used to vacuum excess fluid during scaffold fabrication and improve the scaffold porosity, as well as the bladder-like geometry that is used to manipulate the shape of collagen after fibrogenesis.



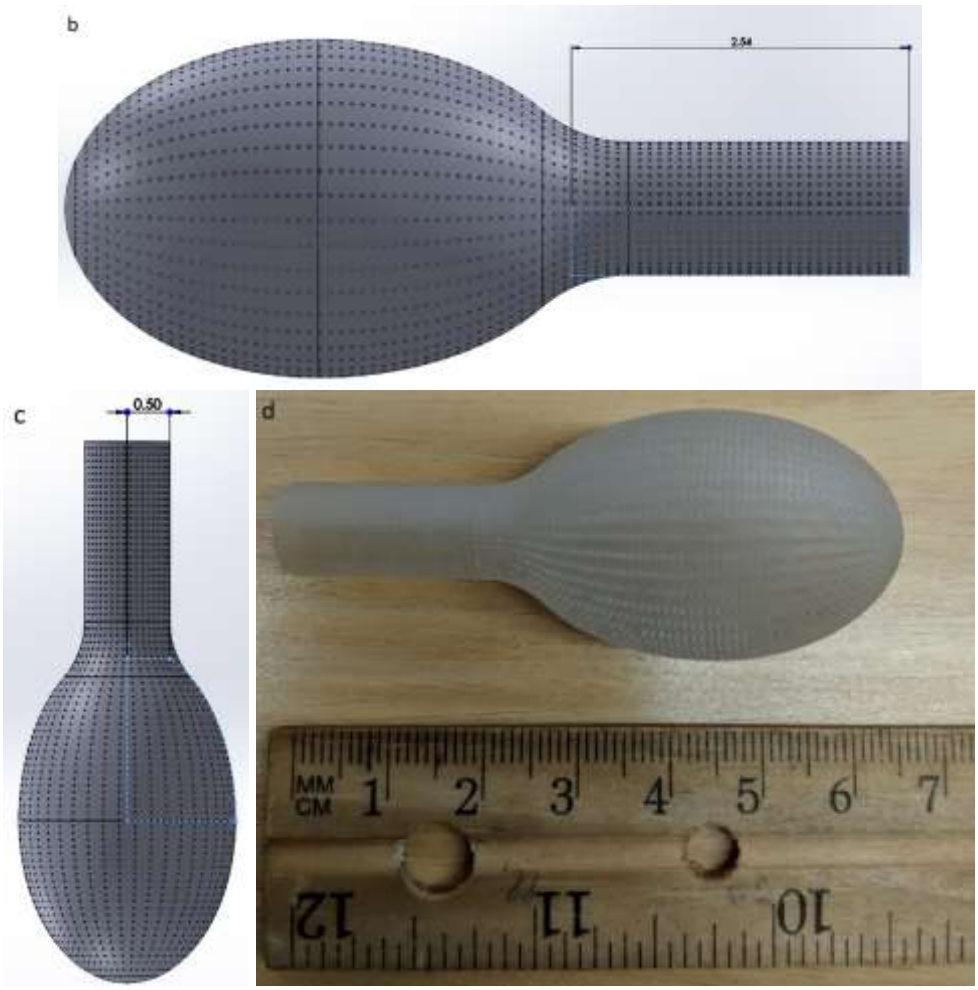
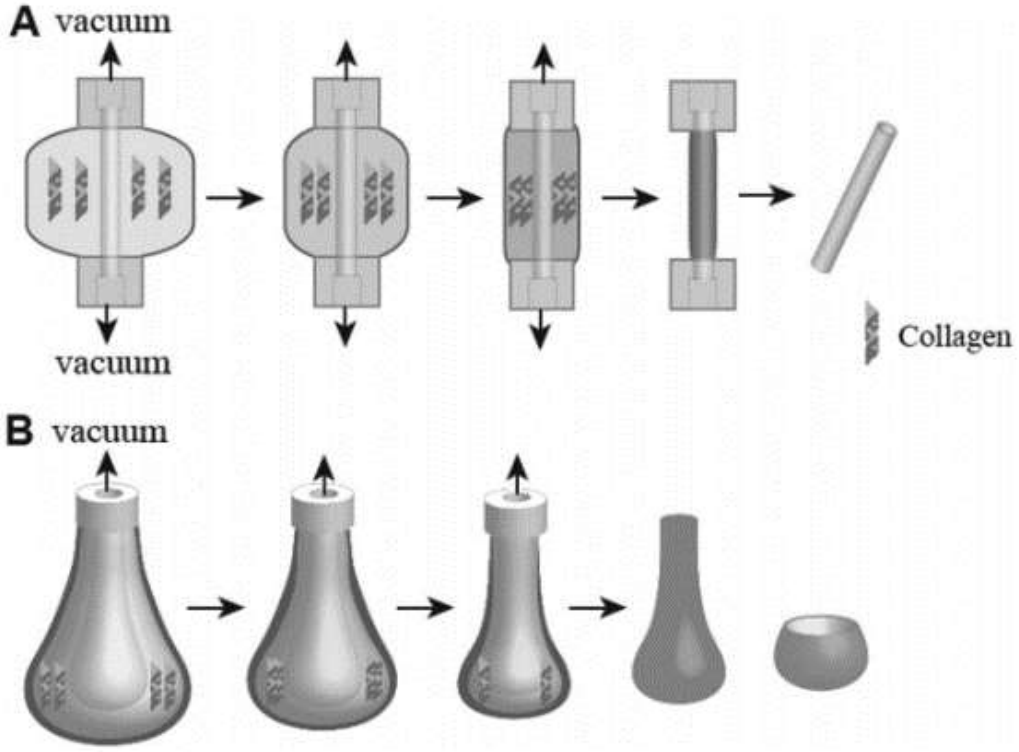


Figure 6: CAD drawing and 3D print of bladder-shaped mold. Dimensions for the balloon section (a), tube section (b), and outer radius (c) are shown. CAD design was performed in SolidWorks™. The microporous structure enables the vacuuming of excess solution and compression of collagen against the mold to form the desired shape. A biocompatible material (MED 610) was used to print the mold (d).

We elected to use collagen for the fabrication of scaffolds and preliminary testing. Collagen would not elicit a foreign body response and would provide intrinsic proteins. The primary concern therefore shifted from an equal weight between mechanical strength and host response to a sole focus on the mechanical strength. Results from mechanical tests would show the necessity of modification to improve the mechanical strength of collagen. We later conducted experiments to observe the baseline performance of collagen.

2.3 Fabrication of Bladder-Like and Ureter-Like Scaffolds

There are various methods for fabricating tissue engineering scaffolds. In the past, we were able to produce tubular and sac-like collagen scaffolds following a method based on the work mentioned in Singh et al **(Figure 7)**. This version does not use a heated mold, but instead uses a 3D-printed mold that collagen compresses against after fibrogenesis, all while excess solution is removed by vacuum. It is a combination of similar techniques used in vacuum thermoforming and stretch-blow molding. Collagen fibrogenesis occurs within the balloon and a vacuum pulls the fibers towards a porous mold (vacuum thermoforming) under the contractile pressure of the balloon (opposite of stretch-blow molding). The fabrication of these scaffolds is dependent on the support provided by the balloon and the internal porous mold that removes excess solution and further condenses collagen fibers on fibrogenesis. [20]



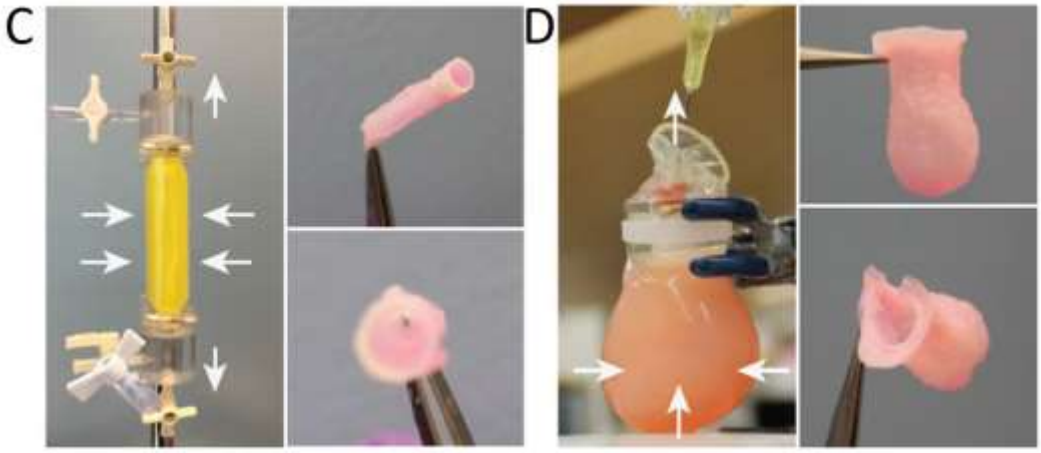


Figure 7: Biomanufacturing of tubular and sac-like collagen scaffolds. Collagen undergoes fibrogenesis in the volume it occupies between the balloon and the mold. Afterwards, collagen compresses against the mold as excess solution is vacuumed out. A schematic of tubular (A) and partial/full sac-like (B) scaffolds displays the process, while the results for the tubular (C) and sac-like (D) scaffolds shows fabrication of the respective shape. [20]

Molds were designed in SolidWorks™ and fabricated by 3D printing. Desired shape, wall thickness, and porosity were considered in the building and designing of CAD models **(Figure 6)**. The 3D Print Lab at the Carnegie Center for Surgical Innovation printed CAD designs using the respective files. A biocompatible material, MED 610, was used for these molds to mitigate the occurrence of a synthetic mold affecting the properties of collagen scaffolds fabricated using one of the molds. We were able to produce bladder-like and tubular scaffolds using older molds **(Figure 8)**. Surface area calculations showed that the latest rendition of the bladder mold would require a lot of collagen solution to produce a scaffold. We were therefore unable to produce a scaffold due to our limited supply of collagen and its financial constraints. The protocol for fabrication of tubular and bladder-like scaffolds would follow the procedures from previous experiments conducted by Singh et al, which are like the steps mentioned earlier regarding vacuum thermoforming and stretch-blow molding. [20]

Figure 8: Collagen scaffolds produced via compression molding. Tubular (a) and bladder-like (b) scaffolds were produced by the process shown in **Figure 7**.

CHAPTER 3: MECHANICAL EVALUATION OF COLLAGEN AS A BIOMATERIAL FOR URINARY TISSUE ENGINEERING

3.1 Introduction

The mechanical properties of biomaterials are pivotal in their ability to function as tissue engineering scaffolds. If a biomaterial is not strong enough to withstand certain conditions, then the scaffold will break. While there are other critical factors to consider, such as the foreign body response to a material, the mechanical strength is a primary concern because it determines how sustainable a scaffold produced from a specific biomaterial can be. Other critical steps such as cellular activity and tissue regeneration cannot be accomplished if the scaffold cannot withstand fluid pressure.

We looked to determine the mechanical strength of collagen fabricated by our method of compression molding. We designed different experiments based on the conditions we believed to be important for a urinary scaffold. Standard tensile tests would give stress/strain profiles and help to determine basic mechanical properties. While the pure strength is important, a bladder will frequently expand and restore its shape. Thus, we deemed that in addition to evaluating the pure strength of collagen, we would also evaluate the stress and strain profile through a hysteresis loop. We also proposed a suture test, where a sample would be sutured on each end and stretched until its breaking point. These tests would help to observe the degree to which collagen retained its mechanical properties.

The mechanical analysis is consistent with our prior work and builds off it. Singh et al. observed the mechanical strength of tubular collagen scaffolds produced from a similar biofabrication chamber by performing standard tensile tests. In our proposed tests, we planned to

test the mechanical strength of collagen strips cut from collagen discs from the designed biofabrication chamber. In addition, the hysteresis and suture tests will give different points of evaluation for collagen.

3.2 Materials and Methods

3.2.1 Collagen Disc Preparation

The main part of the setup for the collagen disc included two molds held and separated by about 3 cm in a Findom finger condom. Each mold had a microporous disc inserted for filtering out excess fluid while keeping collagen entrapped. Luer locks were used to add and remove solution, and to prevent solution from escaping during fibrogenesis **(Figure 9a)**. The locks inserted into the body were used for adding and removing solutions with a syringe, and the ones attached at the top and bottom were used for removing solution through a self-controlled vacuum. A round bottom Erlenmeyer flask was used in a double trap vacuum to collect the excess solution **(Figure 9b)**.

Figure 9: Setup for making a collagen disc. A closer view displays the main section of the setup, with arrows showing directions of moving components (a). A wider view of the setup shows all the components (b). Collagen solution is added through the Luer lock on the side of the upper mold. It undergoes fibrogenesis while encased within the balloon between the two molds. The vacuum runs through the top and bottom Luer locks and vacuums excess solution to a round-bottom flask using a double trap vacuum setup. When the balloon attained an hourglass shape, the top mold was brought down until the sides were parallel.

Compressed collagen discs were made with a mix of collagen (native collagen, bovine dermis 5 mg/mL) with Dulbecco's Modified Eagle's Medium (DMEM, 1X), fetal bovine serum (FBS), and HEPES buffer (1M). A ratio of 88 vol% DMEM, 10 vol% FBS, and 2 vol% HEPES was used to make neutralizing solution for the same amount of collagen. For 5 mL discs, 6 mL of collagen was mixed with the neutralizing solution mix of 5.28 mL DMEM, 0.6 mL FBS, and 0.12 mL HEPES. For 10 mL discs, 11 mL of collagen was mixed with the neutralizing solution mix of 9.7 mL DMEM, 1.1 mL FBS, and 0.22 mL HEPES. The neutralizing solution mix was added to the collagen with both solutions at 4°C and mixed using a motorized pipette. The mixed collagen solution was transferred into the apparatus using a 30 mL syringe. After letting fibrogenesis

complete while leaving the apparatus untouched for an hour, excess fluid was vacuumed by pressing the top mold down when the curvature of the balloon condensed into an hourglass shape.

Collagen discs were removed from the setup and dried briefly. They were transferred in petri dishes to an incubator to vitrify for 20 hours, which helped to further remove excess solution. A fabricated collagen disc before and after vitrification is distinguishable by the pink coloring apparent before vitrification, which comes from the DMEM used for the neutralizing solution **(Figure 10)**. Vitrified collagen discs were sealed in small petri dishes and stored in 4℃.

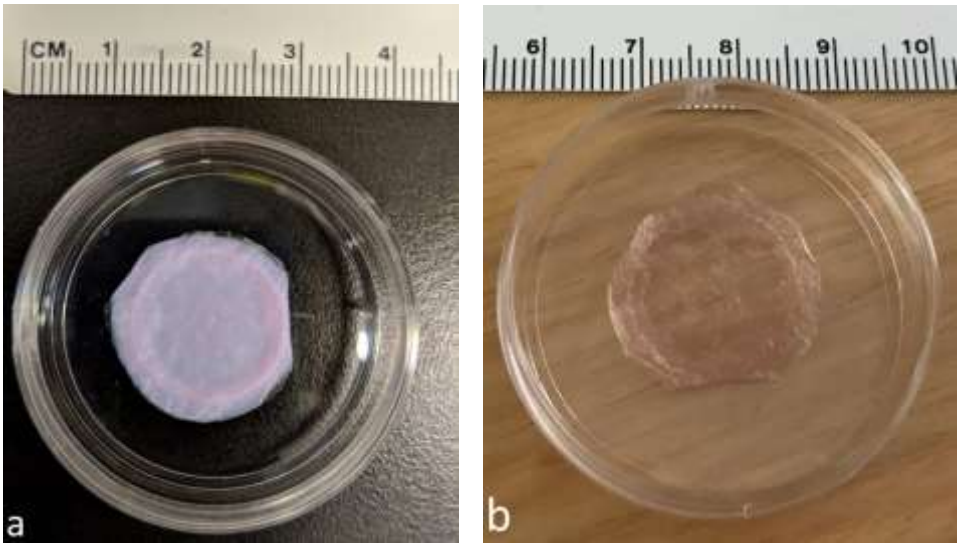


Figure 10: Collagen discs fabricated from the biofabrication chamber. Collagen discs immediately after completion of biaxial compression by vacuum showed slight pink coloring due to the use of DMEM (a). Collagen disc after 20 hours of vitrification were colorless due to the removal of water (b).

3.2.2 Mechanical Testing Experiments

Prior to testing, collagen discs were removed from 4℃ and cut into rectangular strips using a razor blade. Collagen strips remained hydrated in phosphate-buffered saline (PBS) at room

temperature to prepare them for testing. Before putting in the testing apparatus, collagen strips were blot-dried with a Kimwipe and cut into small rectangular strips if needed.

For tensile tests, each sample was put in a paper apparatus that was gripped at each end using tensile grips **(Figure 11)**. Small paper strips with Krazy Glue were used to hold a sample at both ends. The length, width, and thickness were recorded prior to testing. The length was considered as the distance between both ends of collagen in the paper apparatus. Immediately prior to gripping the apparatus, the sample was blotted with PBS. Samples were stretched at various rates from 1-2 mm/min that were established as inputs before the start of a run.

Figure 11: Paper apparatus for holding collagen during tensile and hysteresis tests. Paper ends were held by tensile grips as the upper end was stretched out. Length measurements were recorded prior to making cutouts on the sides to ensure a straight measurement. The sides are cut out in this figure, but during experiments they were cut immediately prior to the start of a run to keep a sample as straight as possible.

For suture tests, samples were threaded and knotted on each end with a suture string. Each knot was held by a tensile grip. The length, considered as the distance between both sutures, was recorded prior to testing. Samples were stretched at various rates from 1-2 mm/min.

Hysteresis testing was performed for vitrified collagen, which had a lower water content and improved porosity. Vitrification was performed to crosslink collagen. The setup was the same as that used for the tensile tests. For this experiment, the sample went through three cycles of stretching and restoring. The max length was set at 30% of the strip length, and the elongation rate was set at 10% of the max length per minute.

Pericardium, a product produced by Coloplast, was tested and compared to collagen as a standard because of its collagen composition. Before testing, it was cut into strips and stored in PBS to maintain freshness. We performed tensile and suture tests to compare the breaking point from each test with that of collagen. The methods for testing pericardium followed the same procedure as the collagen testing.

All tests were performed using the MTS Criterion^{TM} Model 43. A 5N load cell was used to record measurements. For tensile and hysteresis tests, the width, thickness, initial length, and elongation rate were inputted before starting a run. For suture tests, only the initial length and elongation rate were inputted before starting a run. The initial length was measured as the distance between the ends where a strip was held. Data recording concluded when a sample broke or the load (force) exceeded the limit of the load cell. All data was transferred to and analyzed in Microsoft Excel and GraphPad Prism.

3.3 Results and Discussion

3.3.1 Stress and Strain Calculation

The main objective of these experiments was to evaluate and compare the mechanical properties of compressed collagen to the mechanical properties of the bladder of several other

species, but mainly the human bladder. The experimental data obtained would give the results needed to make these comparisons and note the necessary improvements and limitations.

For tensile and hysteresis test results, samples were evaluated based on their stress-strain profile. The stress, given in megapascals (MPa), was calculated by using the formula shown.

$$Stress\ (MPa) = \frac{Load\ (N)}{Cross\ sectional\ area\ (mm^2)} = \frac{Load}{Width\ (mm) * Thickness\ (mm)}$$

The strain, defined as the percentage of how much a sample stretched out from its initial length, was calculated as shown.

$$Strain\ (\%) = \frac{Elongation\ (mm)}{Length\ (mm)} * 100$$

The breaking point of a sample was determined by the ultimate tensile strength (UTS) and ultimate tensile strain, which were defined by the maximum values of stress and strain recorded before a sample completely broke or a run ended.

For suture tests, samples were evaluated based on their load-strain profile. The load was given in gram-force (gf). The cross-sectional area was not necessary to account for because of the expansion and offsetting due to suturing. The breaking point was determined by the ultimate strength and ultimate suture strain, defined as the peak values of load and strain before a sample broke or a run ended.

3.3.2 Mechanical Strength of Collagen

Unmodified collagen displayed qualities of a semi-elastic biomaterial. It exhibited a J-shaped curve in its stress-strain profile from the tensile tests **(Figure 12)**. J-shaped curves show

that initial stretching is easier on a material and requires less stress than longer extensions do. Biological soft tissues commonly exhibit a J-shaped stress-strain curve. As collagen is stretched, fibers straighten and align to provide more strength. Fibers can endure the magnitude of the load at lower strain but not all can endure higher strain. [22]

The tensile curves show that both collagen and pericardium can endure higher forces but are more brittle. Vitrification resulted in a noticeable improvement in the UTS and ultimate strain, which were also slightly than that of pericardium. Both versions of collagen and pericardium endured a constant increase in stress from initial stretching to the breaking point. In contrast, the rabbit ureter is weaker but more flexible as shown in its ability to stretch past 100%. The stress-strain curve resembles a J-shape much more, as the rabbit ureter did not exhibit much stress until around 100% strain. Vitrified collagen is the most suitable choice of the biomaterials tested, but the elasticity would need to be improved for it to be more viable.

The suture curves show more promise in the viability of collagen, specifically vitrified collagen. While unmodified collagen was weak and brittle, vitrification improved the suture retention strength and strain. EDC/NHS crosslinking did not show any beneficial effects, as it decreased the strain of collagen while providing a minimal improvement to the suture strength. Pericardium was much stronger than any other material tested and shared a similar ultimate strain with the rabbit ureter. Based on the suture profiles, vitrified collagen was the most like the rabbit ureter. Improvements to the elasticity and strength would optimize vitrified collagen.

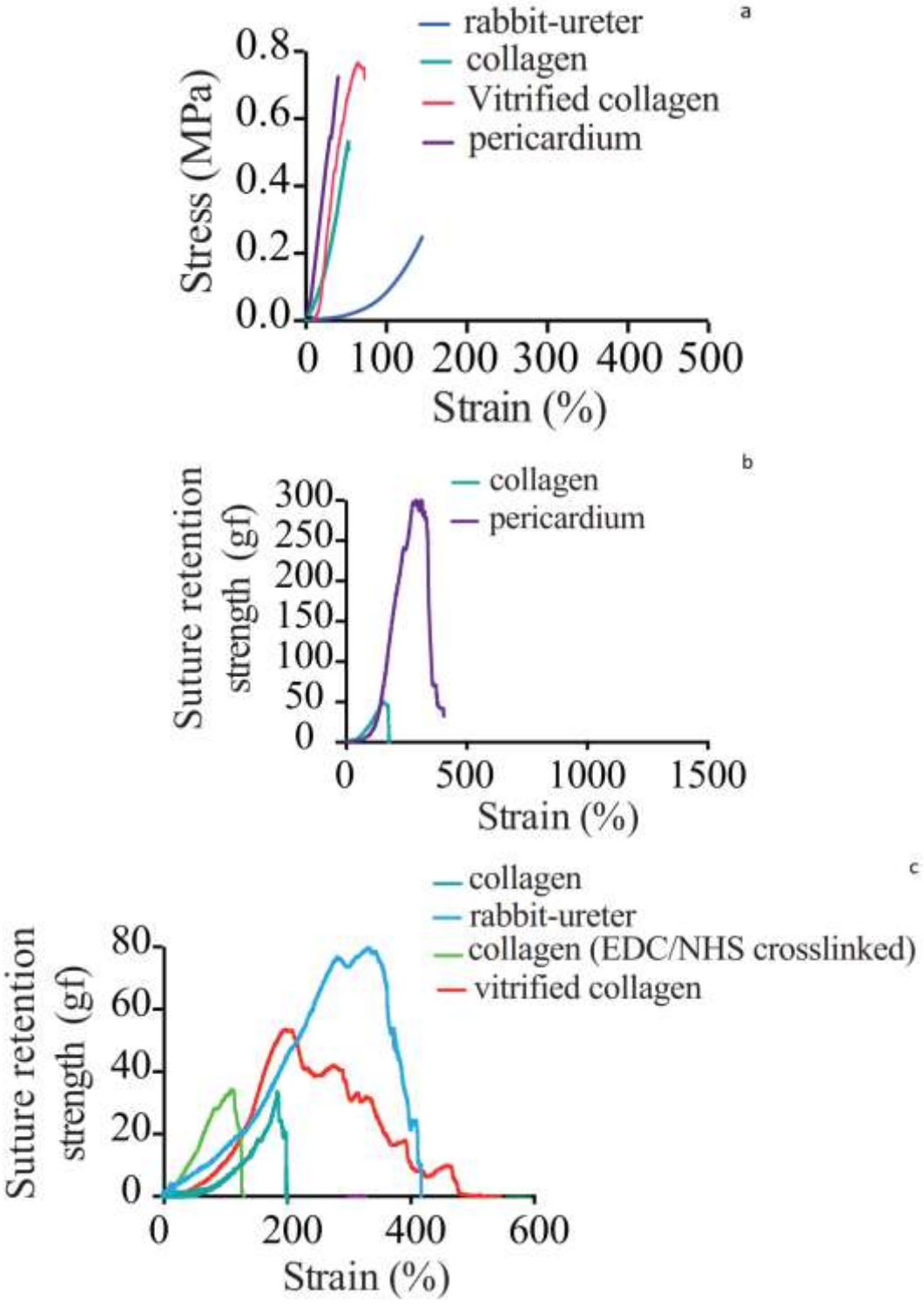


Figure 12: Tensile and suture profiles of materials. Tensile profiles display the standard stress-strain curves up to the breaking points (a). Suture profiles display the mechanical strength and breaking tendencies of materials (b, c). Based on the suture tests, Vitrified collagen shared the most resemblance with the rabbit ureter. None of the tensile curves were like that of the rabbit ureter, although vitrified collagen possessed the highest UTS and ultimate strain. Each material was tested multiple times, but the curves shown are representative of their general behavior.

The hysteresis experiment required a sample that was unlikely to break during several cycles of stretching and restoring. We chose not to test unmodified collagen since the tensile results showed that it only held to around 60% strain. We used vitrified collagen because its lower water content and improved porosity made it more viable. The max length was set at 25% to be safe and ensure that the sample would not tear in the middle of the experiment. For pericardium, the max length was set at 33%. The max length for the rabbit ureter was set at 100%.

The results verified the viscoelastic properties of collagen with multiple time-dependent strain cycles **(Figure 13a)**. A purely elastic material will have complete reversibility, but a viscoelastic material is not completely reversible. Viscoelastic materials exhibit a time delay in moderate resistance to shear flow and absorb energy. The area between the stretching and relaxing curves is known as hysteresis. [23]

Vitrified collagen was able to endure three cycles of stress-strain hysteresis. The low values in stress are a result of the low strain percentage used. These experiments served to observe the behavior of materials rather than evaluate their ultimate strength values. All three materials tested exhibited similar characteristics in that the second and third cycles of hysteresis were nearly identical. The first cycle is typically different as it is when the material is initially stretched out, but memory helps a material to stretch to an identical strain with less exerted force.

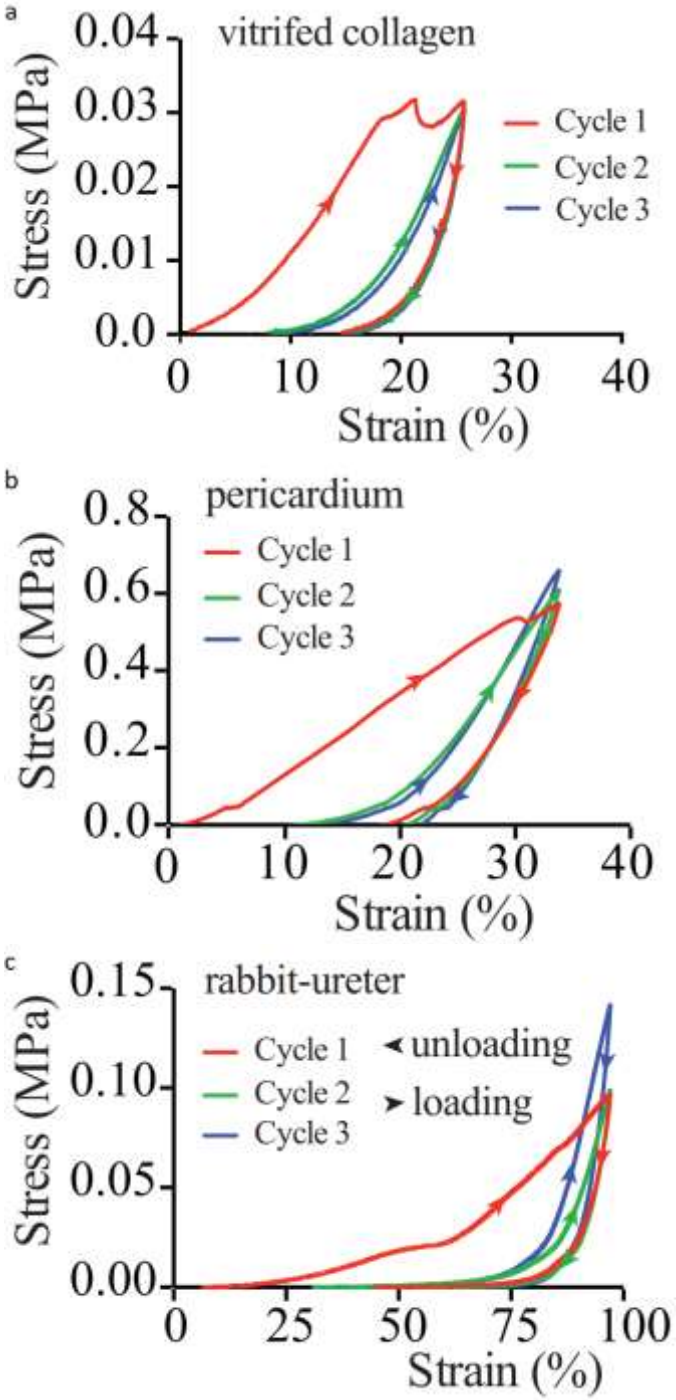


Figure 13: Hysteresis profiles of various materials. Vitrified collagen (a), pericardium (b), and rabbit ureter (c) were stretched to various strain percentages in three cycles of stress-strain hysteresis. Each cycle displays a J-shape curve and loss of energy during relaxation, displaying the viscoelasticity of each material through multiple stress-strain cycles.

3.3.3 Use of Pericardium as a Reference

Pericardium Tutoplast-processed allograft tissue is a commercially available product from Coloplast. It is a decellularized tissue from bovine pericardium and contains collagen. Multi-directional fibers allow the membrane to conform to any shape. It was compared alongside the experimental collagen because it is an industrial biological tissue. Ideally, our method of fabricating collagen scaffolds would produce a variation of collagen as strong as a commercially produced product. [24]

In the tensile and suture tests, pericardium exhibited a J-shape stress-strain curve. Its tensile profile is not indicative of its UTS because it typically exerted a force that was greater than the maximum capacity of the load cell **(Figure 12a)**. Its tensile behavior was still like that of collagen, as stress started to increase almost immediately and noticeably with increased strain. Pericardium also exhibited a higher suture retention strength than collagen **(Figure 12b)**. Its ultimate strength was significantly greater than the ultimate strength of collagen.

Overall, its mechanical properties were much stronger than that of collagen. As a commercial product, it was unsurprising to see it perform well mechanically. The multi-directional fibers are one of many potential factors that contribute to its mechanical strength. Processing techniques were also likely contributors to its strength. The staggering difference in strength showed more about the deficiencies of our fabricated collagen.

3.3.4 Mechanical Limitations

Collagen lacked the mechanical strength that other samples possessed. It did not perform near the level of pericardium and could not achieve 100% tensile strain. Comparisons between the

UTS and ultimate tensile strain of compressed collagen with that of the human, pig, and rat bladder affirm that collagen would not be mechanically viable for any of these animals **(Table 1)**. Collagen barely managed to perform as well as the human bladder. Its UTS and ultimate tensile strain fell short to that of the pig and rat bladder. The ultimate strain did not match that for either of the three animal bladders.

Table 1: Mean (SD) tensile properties of the urinary bladder in rat, pig, and human. (Table from Dahms et al.) [25]

Material	UTS [MPa]	U Tensile Strain [mm/mm]
Rat bladder	0.72 ± 0.21	2.03 ± 0.44
Pig bladder	0.32 ± 0.10	1.66 ± 0.31
Human bladder	0.27 ± 0.14	0.69 ± 0.17

Mechanical strength is a key checkpoint on the path to clinical viability for tissue engineered scaffolds. This method for biofabrication of collagen would not be suitable for the creation of an artificial bladder for either of these mammals because of its lacking mechanical strength. Modifying collagen to increase the mechanical strength is important to proceed with *in vivo* testing and future directions. An improved version of collagen could potentially be a suitable biomaterial choice for a human bladder.

The collagen hysteresis experiment performed utilized vitrified collagen. The tensile results showed that unmodified collagen would not have been sufficient for a hysteresis test and a modified version of collagen would be necessary. We used our results to observe the similarities and differences between vitrified collagen and cross-linked collagen. A hysteresis experiment with unmodified collagen would have been performed at low strain and most likely inconclusive to the overall performance. Nevertheless, it would be good to observe the stress-strain hysteresis profile

of unmodified collagen to observe if it still exhibits viscoelasticity and compare it with other modified versions of collagen.

3.3.5 Potential Challenges

Although the results may infer that collagen is slightly short of being a mechanically viable biomaterial for urinary tissue engineering scaffolds, it is important to note some of the limitations of collagen-based scaffolds from this study and in general. The biofabrication process devised is advantageous because of the easiness of manipulation of 3D shape, porosity, and thickness. However, the results showed that this method does not provide collagen with the mechanical strength necessary to be used for urinary tissue engineering scaffolds.

On top of the lacking mechanical strength, the cost and time investment to produce each scaffold may make this process less effective. Fabricating a collagen scaffold can require a copious amount of collagen, which we experienced with our updated bladder mold. Even if this biofabrication process is quicker than other methods, more thorough analysis of these areas would be needed to make a viable comparison with other processing techniques. The degradation rate of collagen is also of concern, since natural biomaterials are known to degrade faster than synthetic biomaterials. The best way to observe the degradation rate would be through *in vivo* studies.

Biomaterials for tissue engineering in urology have been gaining traction for some time. While they were first used to repair urinary tract segments, they are now being experimented to create 3D scaffolds that can serve as total replacements for patients with deficiencies in their urinary tract. However, one of the biggest problems holding back all types of scaffolds from the next step is the evidence that they can match current urological tissue replacement therapies. Some

significant areas of concern that are hindering the progression of 3D scaffolds are cost, efficiency, manufacturing, and regulation. The biggest concern is the proof of *in vivo* function and viability. Larger genitourinary defects require complex networks that biomaterials have not been shown to have. The urinary system has many other factors that should also be considered, including surrounding mechanical forces, pH, cytotoxic agents, signaling agents, and oxygen levels. The effect of these concerns on the mechanical properties of a replacement scaffold would have to be observed and addressed to evaluate the mechanical viability of a biomaterial for urinary tissue engineering. Even if some factors have a minimal to no effect on mechanical properties, they are important to address to reach the ultimate goal of creating a 3D scaffold that can be applied clinically. [21]

3.3.6 Alternatives and Potential Improvements

In consistency with earlier statements, a stronger material is safer and can handle extreme cases better. Modified collagen could provide the extra strength needed to validate collagen as a viable biomaterial for urinary tissue engineering. Singh et al. modified collagen by adding hyaluronic acid, which resulted in improved strength. [20] We have tested some modifications that we cannot disclose currently, but our results with these experiments have verified that chemical modification is a solution to improving the weak inherent mechanical strength of collagen.

The bladder can stretch in multiple directions, meaning that multidirectional force is a factor in the mechanical analysis. Therefore, a biomaterial should not be completely evaluated based on its uniaxial stretching. Biaxial stress-strain experiments would give a better reflection of a biomaterial for urinary tissue engineering. Collagen discs are compressed biaxially during

fabrication, but we plan to perform biaxial mechanical tests for future samples to observe if there is a difference in mechanical strength between different axes.

The testing conditions for runs that required more time were not the most ideal because samples dehydrated and dried up with time, which affected the material properties. This problem was most prevalent for the hysteresis test, which took about an hour, but could have been a factor in the longer suture and tensile tests. Stretching strips at a rate based on length rather than a constant value may produce more consistent results and will be considered, as larger or thinner strips may dry out quicker than smaller or thicker ones. Furthermore, the apparatus for holding collagen could be improved to reduce the risk of slippage during a run. Dahms et al. used a 'sandpaper sandwich' to improve grip, which we plan to implement in future mechanical tests to observe the effectiveness of sandpaper. [25]

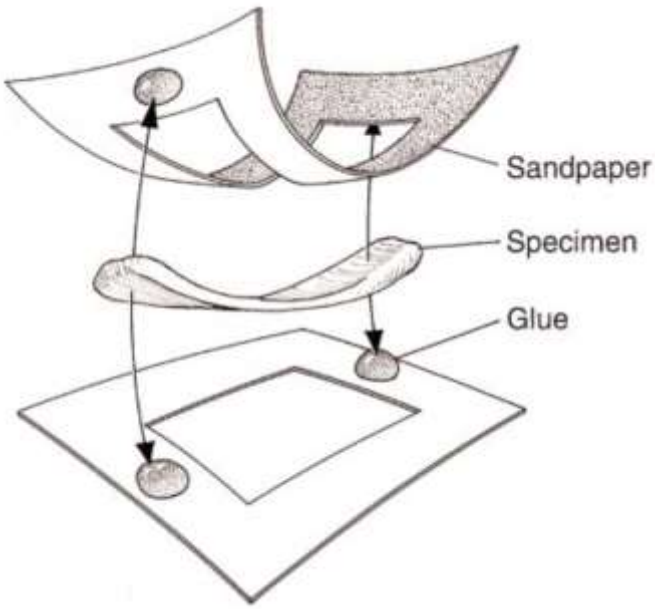


Figure 14: Dahms et al. schematic of the 'sandpaper sandwich' used for tensile testing. Sandpaper helps to prevent slippage of the apparatus from the grips during mechanical testing. We plan to explore this to mitigate slippage that we occasionally experienced. [25]

Our sample size was less than ideal for nearly every experiment, with the potential exception of the collagen suture tests. Due to financial constraints, we limited our experiments to fewer samples. Furthermore, we had to ration collagen discs and solution for other experiments, some of which were unrelated and therefore not mentioned. Production of the sac-like and tubular scaffolds requires a larger volume of collagen because they are larger than discs. With more collagen, we would have been able to produce a larger sample size.

CHAPTER 4: CONCLUSION AND FUTURE OUTLOOK

4.1 Conclusion

Urinary tissue engineering methods have sought to find a way to produce a clinically viable scaffold. One of the key factors in engineering of a scaffold is finding a biomaterial suitable for the conditions of the urinary system. Fabricated scaffolds must be able to resist breakage by pressure from stretching. Collagen has many desirable qualities of a biomaterial for urinary tissue engineering, as it is a natural biomaterial and is biocompatible. Further tuning of mechanical properties is required, so we proposed testing whether collagen fabricated by our biofabrication chamber is mechanically viable to be used for urinary tissue engineering scaffolds.

Our method of compression molding and fibrogenesis allowed us to successfully create microporous collagen scaffolds and shape them as bladder-like and tubular scaffolds. Fabrication of these scaffolds requires a good amount of collagen solution. Furthermore, the viscoelastic properties of collagen were confirmed by its endurance and the stress-strain curves from the hysteresis test of vitrified collagen. However, collagen produced by the compression molding method did not have the sufficient mechanical strength to be used as a biomaterial for urinary tissue engineering scaffolds. Its ultimate strength and stress from both the tensile and suture tests barely matched or was slightly less than the standards for parts of the human urinary system.

Various routes can be taken to improve the mechanical strength of collagen. Structural modification is a frequent solution to improving and modifying specific qualities of a biomaterial. There are many approaches to modification, whether performed through changing the solvent or administering additional treatment. We believe that structural modification can address this issue and is relatively easy to perform. We have already experimented with various modifications to

improve the strength while trying to maintain the biocompatibility of collagen and are still in the process of weighing the options.

4.2 Future Outlook

A primary concern emphasized from the results is the lacking mechanical strength of compressed collagen. Since emphasis was placed on making a mechanically compliant biomaterial, this goal should be completed before moving onto further steps. Physical and chemical modifications are some of the simplest and more straightforward methods to address this issue. Many physical and chemical modifications have already been considered and experimented with. The goal here is to find the modification that will improve mechanical strength the most while retaining optimal biocompatibility.

Following the confirmation of the mechanical viability of a biomaterial, one of the next steps would be to evaluate the performance of compressed collagen scaffolds under urinary flow conditions. To do this, we plan to create a urodynamic flow chamber, which would hold a scaffold and power urinary flow through a chamber. By simulating urinary flow through the chamber, we can use data acquisition to observe pressure values along a scaffold. We would evaluate the performance of both bladder-shaped and tubular scaffolds to determine if certain areas of a particular scaffold are weaker and require more attention.

In future work, we plan to evaluate cell-seeded collagen scaffolds in small animal models to study their *in vivo* biomechanical performance. Small animal models would also be used to observe scaffold ability to support cellular activity. We hope to eventually utilize our

biofabrication method to create collagen scaffolds for other purposes, such as the small intestine, blood vessels, and nerve conduits.

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CURRICULUM VITAE

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SUMMARY

Seeking to apply extensive laboratory research experience to a full-time research position in biotechnology. Proficient in working with biomaterials, mammalian cell culture, and DNA/RNA/protein purification. Passionate engineer and researcher who focuses on progress, contributing to a bigger cause, and making a difference.

EDUCATION

Johns Hopkins University **Baltimore, MD**
Master of Science in Engineering in Chemical & Biomolecular Engineering **Aug 2017 – Jun 2018**

- "Biofabrication of Collagen Scaffolds for Reconstruction of Urinary Tissues" thesis with Dr. Anirudha Singh
- Coursework: Advanced Thermodynamics in Practice, Biomaterials II, Design: Pharmacokinetics/Dynamics, Interfacial Nano Systems, Introduction to Polymeric Materials, Polymer Design and Bioconjugation

Bachelor of Science in Chemical & Biomolecular Engineering **Aug 2012 - May 2017**
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SKILLS AND INTERESTS

Lab Techniques: Proficient in Cell Culture, DNA+RNA Isolation & Purification, PCR/RT-PCR; Familiar with Chromatography, Immunohistochemistry, Microscopy, PAGE/SDS-PAGE, Spectrophotometry, Western Blot
Programming Languages: Proficient in MATLAB; Familiar with Java
Software: Proficient with GraphPad Prism, SolidWorks; Familiar with AspenPlus, LabVIEW
Foreign Languages: Basic Japanese, Korean
Interests: Basketball, Card Games, Technology, Tennis, Urban Dance

RESEARCH EXPERIENCE

Johns Hopkins University School of Medicine, Singh Lab **Baltimore, MD**
Research Assistant, Undergraduate & Master's Program **Aug 2016 – Present**

- Conducted research for Master's thesis to design and fabricate collagen-based scaffolds for urinary tissue engineering, emphasizing biocompatibility and mechanical strength
- Created numerous scaffolds utilizing a unique biofabrication setup that included self-made 3D-printed models designed in SolidWorks
- Measured and analyzed mechanical properties of biomaterials and mammalian urinary tract components
- Performed basic cell culture tasks with mesenchymal stem cells (MSCs), smooth muscle cells (SMCs), and human urothelial cells (hUCs) and bench tasks for RNA extractions

Publications: Singh A, Lee D, **Jeong H**, Yu C, Li J, Fang C, Sabnekar P, Yoshida T, Sopko N, Bivalacqua T. Tissue-Engineered Neo-Urinary Conduit from Decellularized Trachea. *Tissue Engineering*. 2018.

Johns Hopkins Institute for NanoBioTechnology, Wirtz Lab **Baltimore, MD**
Research Assistant **Aug 2014 – Oct 2015**

- Studied the interaction between nuclear lamina and chromosomes in fibroblasts cultured on various stiffness hydrogels

- Characterized fibroblasts by gene expression and purity based on mRNA microarray and spectrophotometry data
- Performed high-throughput single-cell imaging using confocal microscopy to study cell shape, migration, and mechanosensing transcription factors
- Maintained cells by culturing and passaging for uses in characterization and imaging

Eyegenix, LLC **Honolulu, HI**
Summer Research Intern **May 2013 – Aug 2013**

- Conducted tissue culture research project on corneal wound healing, working with human corneal-limbal epithelial cells
- Characterized cells by immunostaining for stem cell markers and assessed growth kinetics
- Developed an *in vitro* corneal wound healing model to support development of future products

WORK EXPERIENCE

Johns Hopkins University **Baltimore, MD**
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- Assisted Senior Lecturer Dr. An Goffin in her undergraduate course "Dynamic Modeling and Control"
- Taught 60 students weekly as the lead teaching assistant for the course
- Organized and led review sessions before exams to provide additional support for students